Reduced Nerve Blood Flow in Diabetic Rats: Relationship to Nitric Oxide Production and Inhibition of Aldose Reductase

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This study examined links between impaired nitric oxide production in the sciatic endoneurium, nerve blood flow, and polyol pathway flux, to test the hypothesis that reduced nerve blood flow might be compromised by competition for NADPH between aldose reductase and nitric oxide synthase. Sciatic nerves of streptozotocin-diabetic rats showed reduced laser Doppler flux (by 51 % or 63 %; both $p<0.05$)—indicative of reduced nerve blood flow—and reduced motor nerve conduction velocity (17 % in two experiments; $p<0.05$). Acute interruption of nitric oxide production in the sciatic nerves of control rats, via endoneurial injection of Nω-nitro-D-arginine methyl ester (L-NAME), caused a local reduction (of 64 %; $p<0.001$) in nerve Doppler flux. This was reversed by either L-arginine or sodium nitroprusside. The response to L-NAME was greatly reduced in diabetic rats (only 22 % reduction; $p<0.01$), though both L-arginine and SNP caused marked increases in flux. Chronic inhibition of aldose reductase in diabetic rats (with either sorbinil or imirestat at a range of doses) had little effect on resting sciatic nerve Doppler flux, though both inhibitors normalized conduction velocity. Both aldose reductase inhibitors reduced sorbitol pathway intermediates in a dose-related manner. These findings do not support the proposition that aldose reductase inhibitors normalise conduction velocity by mechanisms dependent upon either normalization of endoneurial nitric oxide or nerve blood flow. Instead, a mechanism based upon more direct effects on axon or Schwann cell function is favoured. © 1998 John Wiley & Sons, Ltd.

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Introduction

Early conduction velocity deficits in the nerves in diabetes mellitus may signal the beginnings of neurological dysfunction leading to the development of diabetic neuropathy. This is supported by evidence in both patients[1–3] and streptozotocin-diabetic rats.[4,5] These early changes may derive from hyperglycaemia via exaggerated flux of glucose through the polyol pathway (see Masson and Boulton,[6] and Tomlinson[7] for review), though exactly how activity in this pathway is coupled to action potential conduction remains to be determined. One hypothesis suggests that decreased nerve blood flow provokes the conduction impairment and this is supported by data from animal studies, including the parallel development of the two phenomena in diabetic rats;[8,9] the development of a similar conduction impairment in centrally hypoxaemic non-diabetic rats[10] and the prevention of the conduction deficit in diabetic rats by a range of drugs which share the capacity to increase peripheral blood flow as their only common denominator.[11–15] Direct endoneurial microinjection of drugs, which manipulate the production and effect of nitric oxide, indicates strongly that nerve blood flow in normal rats is heavily dependent on tonic nitric oxide production and that the latter is virtually absent in diabetic rats.[16]

The conduction deficit in diabetic rats is prevented by inhibition of aldose reductase, the first enzyme of the polyol pathway.[17,18] This enzyme uses NADPH as a proton donor for the conversion of glucose to sorbitol and it is probable that a large increase in flux through this enzyme in diabetes consumes large amounts of NADPH, possibly compromising other reactions which depend on the same cofactor. This may form a mechanism for reduced synthesis of nitric oxide, whose production also uses NADPH, and one aim of our study was to determine whether inhibition of aldose reductase could reinstate tonic nitric oxide production in the sciatic endoneurium. This has had to be done in laboratory animals. The local influence of nitric oxide in the sciatic

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endoneurium was examined, as in our previous study,[16] by endoneurial injection of Nω-nitro-d-arginine methyl ester (l-NAME) while monitoring local nerve blood flow via a laser Doppler velocimeter. Following this responses to either l-arginine or sodium nitroprusside were studied.

Early experiments in this study indicated effects of aldose reductase inhibition at variance with some of those reported in the literature, so that the study was broadened to test dose dependence of the effects of inhibitors on sciatic nerve Doppler flux and to verify their effect on nerve conduction velocity.

Methods

Induction of Diabetes, Treatments, and Functional Measurements

This investigation comprised two self-contained studies. Both used male Wistar rats of weight 320–400 g (Charles River, UK), which were randomly assigned to experimental groups. Diabetes was induced by a single injection of streptozotocin (STZ; 55 mg kg^{-1} body weight, i.p.), freshly dissolved in sterile saline (0.9 % w/v) and given in the morning following an overnight fast. Thereafter all groups were allowed free access to standard laboratory diet and water. Three days later diabetes was confirmed in STZ-treated rats when reflectance photometry of tail blood revealed glucose concentrations in excess of 15 mM.

In the first study four diabetic groups were treated with the aldose reductase inhibitor (ARI), imirestat,[19–21] at a range of daily doses (see Table 1), by gavage. Treatment was begun 3 days after STZ and maintained for 4 weeks. Untreated control and diabetic groups were maintained in an identical manner. At the end of the treatment regime rats were anaesthetized in mixed batches (single intraperitoneal injections of sodium pentobarbitone at 50 mg kg^{-1} body weight (Sagatal, Rhone Merieux, UK) and diazepam (2 mg kg^{-1}, Diazemulus, Dumex, UK)), for measurement of sciatic nerve MNCV and laser Doppler flux exactly as described elsewhere.[22] The carotid artery was cannulated to monitor systemic arterial pressure (pressure transducer Type 4327 L221; Bell and Howell, Colnbrook, Berkshire, UK). Throughout all experimental procedures body temperature was maintained at 37 °C via a rectal temperature probe connected to a heated homeothermic mat.

The second study was similarly organized, but comprised four groups: untreated controls and diabetic rats, plus two diabetic groups given the ARI, sorbinil (25 or 60 mg $kg^{-1}day^{-1}$ p.o.), by gavage. The lower dose was that commonly employed in studies to antagonize conduction slowing with this drug in diabetic rats[18,23,24] and the higher dose was included to guarantee as far as possible cessation of flux through the pathway. At the end of this protocol the rats were anaesthetized (as above) for measurement of conduction and nerve Doppler flux, but flux responses were also studied to injection of drugs designed to manipulate acutely endoneurial production of nitric oxide. These approaches are described in detail elsewhere.[16] In brief, the rationale was endoneurial infusion of l-NAME (1 nmol in 0.5 μl), made over 2 min, to inhibit the production of nitric

Table 1. Baseline data from both studies: body weight, final plasma glucose, systemic arterial pressure, sciatic nerve Doppler flux, and motor nerve conduction velocity (MNCV)

Group	Body weight (g)		Plasma glucose (mM)	Systemic arterial pressure (mmHg)		Nerve Doppler flux (arbitrary units)	MNCV ($m.s^{-1}$)
	Initial	Final		Systolic	Diastolic		
Imirestat study							
Control (7)	308 ± 12	402 ± 18	9.1 ± 0.7	147 ± 19^{a,x}	107 ± 8^{x,a}	216 ± 26^{x}	51.0 ± 7.0^{a}
Diabetic (7)	314 ± 16	321 ± 22	31.5 ± 4.4	120 ± 13^{y}	89 ± 10^{y}	105 ± 13^{y}	42.5 ± 4.5^{b}
Imirestat-diabetic 0.5 mg kg^{-1} (5)	303 ± 8	292 ± 16	31.8 ± 6.5	124 ± 8^{b}	93 ± 8^{b}	100 ± 20^{y}	NM
Imirestat-diabetic 1 mg kg^{-1} (5)	313 ± 27	310 ± 47	30.2 ± 1.7	115 ± 18^{y}	88 ± 16^{y}	90 ± 15^{y}	50.9 ± 8.8^{a}
Imirestat-diabetic 5 mg kg^{-1} (5)	313 ± 12	335 ± 29	29.1 ± 9.2	130 ± 15	99 ± 11	103 ± 18^{y}	NM
Imirestat-diabetic 10 mg kg^{-1} (5)	312 ± 25	296 ± 35	35.5 ± 6.7	116 ± 10^{y}	90 ± 10^{b}	106 ± 10^{y}	NM
Sorbinil study							
Control (12)	358 ± 16	490 ± 29	9.0 ± 1.2	120 ± 14^{a}	107 ± 12^{a}	203 ± 44^{a}	52.6 ± 7.8^{a}
Diabetic (11)	360 ± 19	285 ± 43	46.0 ± 4.5	106 ± 13^{b}	90 ± 15^{b}	75 ± 30^{b}	43.5 ± 6.3^{b}
Sorbinil-diabetic 25 mg kg^{-1} (11)	361 ± 10	300 ± 34	46.6 ± 4.6	120 ± 12^{a}	104 ± 12	110 ± 42^{b}	47.5 ± 4.7
Sorbinil-diabetic 60 mg kg^{-1} (9)	345 ± 10	331 ± 42	44.6 ± 6.1	121 ± 20	92 ± 15^{b}	102 ± 57^{b}	48.8 ± 4.7^{a}

Data are mean ± 1 standard deviation and were analysed by one-way analysis of variance with Duncan's multiple range tests; $p<0.05$ ^{a} vs ^{b} and $p<0.01$ (^{x} vs ^{y}).
NM, not measured. Massive differences in body weight and blood glucose were not tested.

oxide. In controls this produced a brisk reduction in nerve Doppler flux (see Figure 1), which was taken to indicate the presence—prior to the infusion—of tonic endoneurial vasodilator nitric oxide. Approximately 30 min later l-arginine (100 nmol in 0.5 μl) was infused in some of the rats to reverse the effect of l-NAME. In the remaining animals, l-NAME was followed after about 30 min with SNP (10 nmol in 0.5 μl) to examine the response of the guanylate cyclase system to a nitrodonor. Both of these infusions were also made over 2 min.

All drugs were obtained from Sigma Chemical Company (UK) unless indicated otherwise in methods.

Determination of Nerve Glucose, Sorbitol, and Fructose

The left sciatic nerves were rapidly removed, weighed immediately, frozen at −70 °C and then freeze-dried for measurement of dry weight and assay of sugars and polyols by gas chromatography exactly as described elsewhere.[25]

Statistical Analysis

All data are presented as means ± 1 standard deviation. Acute drug effects were evaluated by comparison within animal (effect versus baseline) using paired *t*-tests. Single comparison between groups used one-way ANOVA with Duncan's multiple range tests where $F<0.05$.

Results

Body Weight, Blood Glucose, and Mean Arterial Pressure

Data showing final blood glucose concentrations, changes in body weight and resting mean arterial pressure for all groups in both studies are summarized in Table 1. Diabetic rats were hyperglycaemic and of lower body weight than age-matched controls. Neither ARI affected glycaemia or body weight. The systolic and diastolic arterial pressures of diabetic rats were lower than those of age-matched controls. Imirestat was without effect on arterial pressure, but the diabetic group on sorbinil treatment at 25 mg kg^{-1} had slightly increased systolic pressures ($p<0.05$). This effect was not seen at 60 mg kg^{-1} (Table 1).

Resting Nerve Doppler Flux and Motor Nerve Conduction Velocity

In both studies, the resting sciatic nerve Doppler flux was significantly lower in diabetic rats compared to that of untreated controls (Table 1). The reductions were comparable, with falls of 51 % in the imirestat study and 63 % in the sorbinil study, the small difference possibly being related to slightly more severe diabetes in the sorbinil study (see plasma glucose and body weight data in Table 1). Imirestat, irrespective of dose, was without effect on sciatic nerve laser Doppler flux, though at 1 mg kg^{-1}day^{-1} it normalized MNCV in the

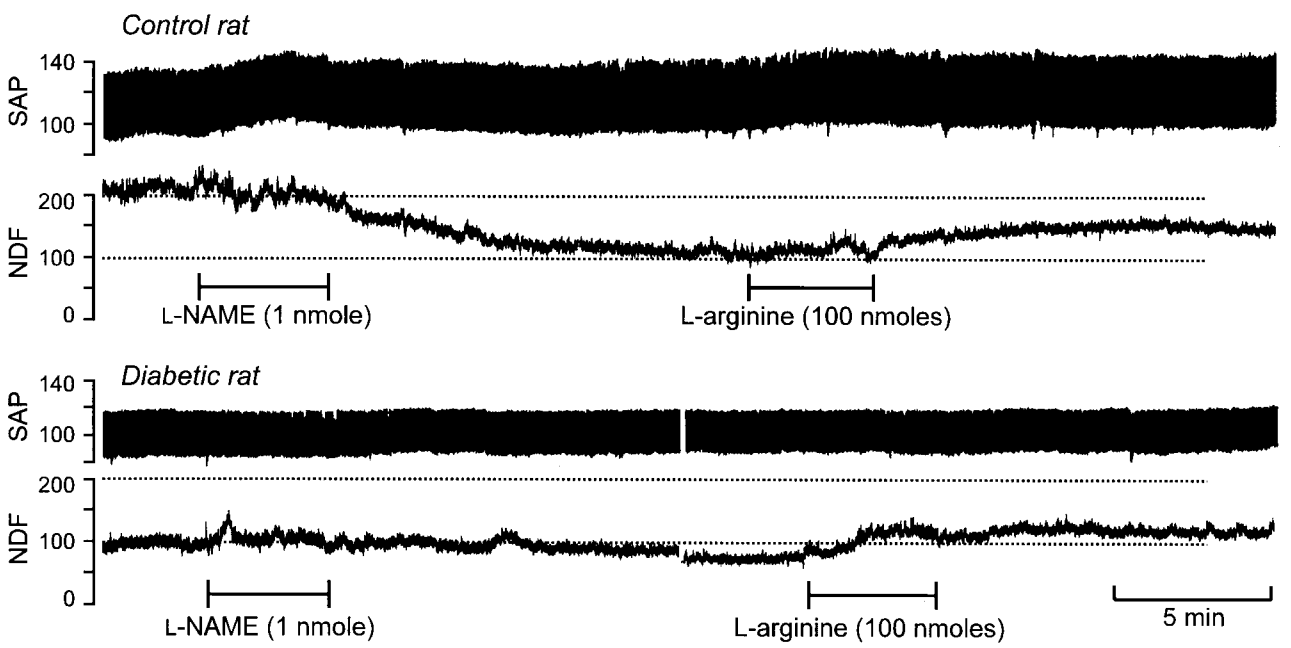

Figure 1. Representative traces from a control and a diabetic rat, showing systemic arterial pressure (SAP in mmHg) and sciatic nerve laser Doppler flux (NDF in arbitrary units) responses to endoneurial injections of l-NAME to inhibit nitric oxide synthase, followed by l-arginine to reverse the blockade. The breaks in the diabetic rat record were caused by a change of micropipette

same nerve (MNCV was not measured at the other doses). Imirestat was effective as an ARI as judged by the normalization of nerve sorbitol at 1 mg kg^{-1} and of fructose at 5 mg kg^{-1} (see Table 2).

Sorbinil treatment failed to prevent the diabetes-induced reductions in nerve Doppler flux at either dose, though there was a trend towards some attenuation (Table 1). However this was not significant, and it did not show any dose-dependence or any relation to nerve sorbitol or fructose levels (Table 2). Both doses of sorbinil attenuated the diabetes-induced reduction in MNCV. At the higher dose of sorbinil, MNCV was not statistically significantly different from that of age-matched controls.

Modulation of Sciatic Nerve Doppler Flux by l-*NAME,* l-*arginine, and SNP*

Figure 1 shows representative traces from control and diabetic rats (the group data in Figure 2 authenticate that they are typical responses), which illustrate the way in which these drugs indicate the status of vasoactive nitric oxide in the sciatic endoneurium. The blockade of nitric oxide production by the microinjection of l-NAME reduced nerve Doppler flux in control rats by 62 % ($p<0.001$; Figure 2). The subsequent administration of l-arginine returned flux towards basal levels for controls and administration of SNP increased flux above pre-l-NAME basal levels in some rats (Figures 1 and 2). The response to l-NAME in diabetic rats was greatly reduced, compared with that seen in controls. The increase in flux in diabetic rats in response to l-arginine was closer to that of controls than was the l-NAME response and the absolute flux change in response to SNP was similar in both groups. There was some evidence of attenuation by sorbinil (at 25 mg kg^{-1}) of the diabetes-induced changes in l-NAME and l-arginine responses (Figure 2), though these effects were not seen at the higher dose.

The endoneurial injections had little systematic effect on systemic arterial pressure. As is shown in Figure 1, control rats frequently showed small pressor responses to the injection itself, but these had always subsided by the time the change (in response to l-NAME, l-arginine or SNP) in nerve Doppler flux was measured. Similar brief pressor responses were reported in our previous study and were also seen with microinfusions of saline;[16] hence they are probably pressure artefacts associated with the infusion itself. In general, such responses were absent in diabetic rats (Figure 1).

The lower dose of sorbinil was similar in its effect on sciatic nerve sorbitol and fructose to the lowest dose of imirestat (Table 2). The higher dose reduced the nerve levels of both metabolites, so that they were indistinguishable from those of non-diabetic rats.

Table 2. Sciatic nerve levels of sugars and sorbitol (nmoles/mg dry nerve) for both studies

Group	Glucose	Sorbitol	Fructose
Imirestat study			
Control	9.6 ± 4.7^{x}	$0.4 \pm 0.3^{y,a}$	1.8 ± 0.5^{y}
Diabetic	42.0 ± 4.7^{y}	10.3 ± 3.6^{x}	19.2 ± 3.1^{x}
Imirestat-diabetic 0.5 mg kg^{-1} (5)	37.3 ± 7.3^{y}	$4.4 \pm 4.7^{y,b}$	11.9 ± 0.89^{y}
Imirestat-diabetic 1 mg kg^{-1} (5)	48.8 ± 1.3^{y}	0.2 ± 0.2^{y}	3.1 ± 1.5^{y}
Imirestat-diabetic 5 mg kg^{-1} (5)	41.6 ± 11.7^{y}	0.1 ± 0.0^{y}	1.2 ± 0.5^{y}
Imirestat-diabetic 10 mg kg^{-1} (5)	41.8 ± 9.4^{y}	$0.1 \pm 0.1^{y,a}$	1.5 ± 0.8^{y}
Sorbinil study			
Control (12)	10.7 ± 3.8^{x}	$0.6 \pm 0.8^{a,x}$	4.0 ± 1.5^{x}
Diabetic (11)	56.3 ± 15.0^{y}	10.5 ± 5.2^{y}	$27.1 \pm 6.7^{a,y}$
Sorbinil-diabetic 25 mg kg^{-1} (11)	63.9 ± 9.2^{y}	$2.7 \pm 4.4^{x,b}$	$14.8 \pm 8.0^{b,y}$
Sorbinil-diabetic 60 mg kg^{-1} (9)	61.2 ± 13.3^{y}	1.0 ± 0.3^{x}	1.6 ± 2.1^{x}

Data are mean ± 1 standard deviation (*n* numbers as for Table 1) and were analysed by one-way analysis of variance with Duncan's multiple range tests. Within each study $p<0.05$ (^{a} vs ^{b}) and $p<0.01$ (^{x} vs ^{y}).

Discussion

This investigation has confirmed that diabetic rats show a reduction of about half in sciatic nerve Doppler flux, a measure of average nerve blood flow. There has been lengthy debate in the literature on the validity of nerve Doppler flux as an index of nerve blood flow under these circumstances and the reader is referred elsewhere for the arguments.[14,26–28] It is certainly true that comparison of baseline Doppler flux data derived from sciatic nerves of different animals can be defended only on pragmatic grounds and must be made with great care. The pragmatic defence derives from the fact that the proportional reduction in the nerve Doppler flux in diabetic rats is similar to that seen with methods which measure flow in more absolute terms.[14,26–28] In the present investigation, the use of Doppler flux enabled the continuous monitoring of changes, within animal, in response to acute interventions and none of the other methods allow this. Thus, it is defensible to conclude from this work that nerve blood flow is indeed reduced in diabetic rats; that acute endoneurial interruption of nitric oxide synthesis abruptly halves nerve blood flow in control rats, but that such an intervention has little effect in diabetic rats. It is striking that the basal level of Doppler flux in the nerves of diabetic rats is similar to that seen in controls after endoneurial injection of l-NAME. Further, the lack of effect of l-NAME in nerve of diabetic rats seen in our previous studies[16,22] and the very modest reduction seen here, suggest that the obviously powerful vasodilator tone derived from endoneurial nitric oxide is lacking in the nerves of diabetic rats.

Alternatively, it could be argued that possible non-linearity in the Doppler signal could preclude resolution of decrements below basal level in diabetic rats or the post-l-NAME level in controls. This is unlikely, however, because we have seen that endoneurial injection of a

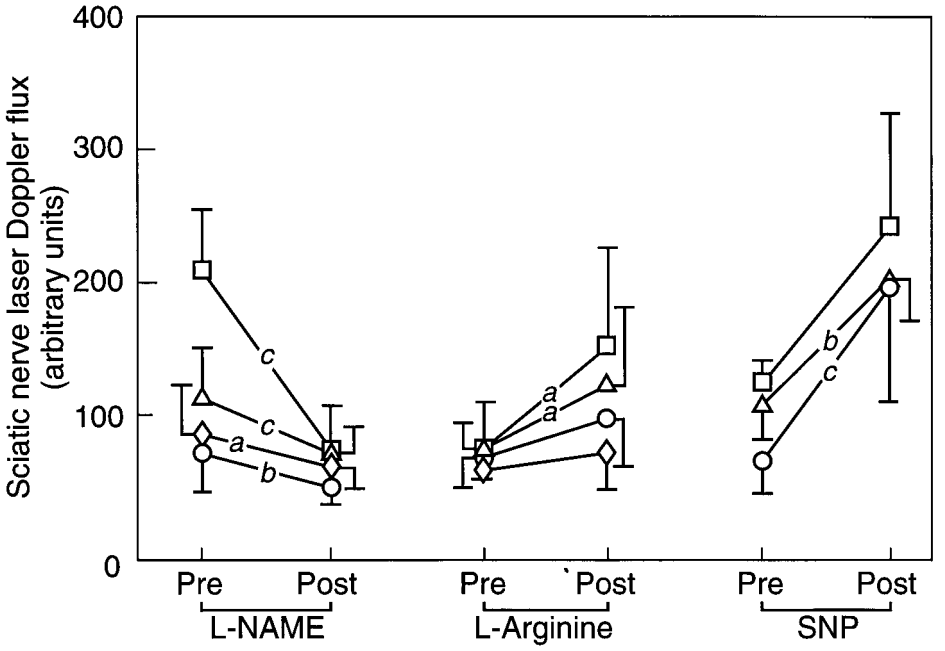

Figure 2. Group responses of sciatic nerve Doppler flux to endoneurial L-NAME ($n = 11$ or 12, except for 60 mg kg^{-1} sorbinil, where $n = 6$) followed by either L-arginine ($n = 5$ or 6) or sodium nitroprusside (SNP; $n = 5$). SNP was not used in the group given 60 mg kg^{-1} sorbinil; □ controls, ○ untreated diabetic rats, △ 25 mg kg^{-1} sorbinil, ◇ 60 mg kg^{-1} sorbinil. Significance of changes in flux (paired *t*-tests) are indicated by the letters, so that $a = p<0.05$, $b = p<0.01$, $c = p\,0.001$. Unmarked lines denote insignificant changes

vasoconstrictor (phenylephrine) in diabetic rats further reduces the signal by about 50 %;[29] thus the system could resolve decrements in Doppler flux in diabetic rats if they were to occur with l-NAME.

The notion that endoneurial blood flow in diabetic rats is heavily dependent on vasodilator tone from nitric oxide is not contentious. Topical application of an inhibitor of nitric oxide synthase also reduces nerve blood flow[30] and, as stated earlier, we have seen the effect of l-NAME in previous studies.[16,22] The present findings do not, however, give strong support to the notion that reduced synthesis of nitric oxide in the nerves of diabetic rats derives from increased flux through aldose reductase. Certainly, there appeared to be a trend towards an increase in both basal flux and in the response to l-NAME with the lower dose of sorbinil, but it is difficult to ascribe much importance to this, since the effect was not statistically significant. Additionally, the effect of the low dose of sorbinil bore no relation to the degree of inhibition of the pathway—as indicated by the levels of sorbitol and fructose—and the effect was not augmented by increased dosage and increased inhibition. Since the participation of nitric oxide in maintenance of nerve blood flow is clear, any important involvement of aldose reductase in impairment of nitric oxide production in diabetes would have been reflected in an increase in nerve Doppler flux with imirestat treatment. That such an effect did not occur is absolutely clear. Our dose–response curve achieved normalization of nerve sorbitol with the top three doses and normalization of nerve fructose with the top two. Thus, we would argue that there is no serious involvement of aldose reductase in either endoneurial nitric oxide production or in the reduction of nerve blood flow associated with experimental diabetes in rats. This is, of course, at variance with observations from another laboratory[28,31] and we cannot explain this; our case rests upon the absolute clarity of our findings.

The present findings indicate that the deficit in nitric oxide in the nerves of diabetic rats appears to be a clear deficiency of production, in that the change in Doppler flux on injection of SNP was of identical proportions in both control and diabetic rats. This suggests that the relevant guanylate cyclase system is unaffected by diabetes. However, the fact that a substantial dose of l-arginine did not restore normal flux in diabetic rats, while it did in controls, suggests that there is a second factor (in addition to impaired nitric oxide production) contributing to the nerve ischaemia in diabetic rats. Posible candidates are endothelin,[15,30,32] angiotensin II[33,34] or deficient dilator prostanoids.[35–37]

The final area of contention in relation to this study is the proposition that prevention or correction of nerve ischaemia is an absolute prerequisite for prevention or correction of the motor nerve conduction deficit.[28,38] Since we were able to prevent the development of reduced MNCV by treatment of diabetic rats with either of these aldose reductase inhibitors without an effect on nerve Doppler flux, we argue that the conduction deficit cannot be causally dependent on the blood flow deficit. Indeed, we have evidence that the reduction in nerve blood flow in diabetic rats is largely derived from muscle wasting, because wasting due to food restriction in non-diabetic rats also reduces nerve Doppler flux, and

muscle-bulking with chronic clenbuterol has the opposite effect (in spite of the fact that acute β-adrenoceptor stimulation reduces nerve blood flow).[39] It is possible that protein catabolism produces endogenous inhibitors of nitric oxide synthase[40,41] and this might be expected to compromise a local circulation that is heavily dependent upon nitric oxide for vasodilator tone.

In conclusion, this study provides a clear solution to the hypothesis put forward in the introduction—aldose reductase is not primarily responsible for reduced endoneurial blood flow in the sciatic nerves of diabetic rats and normalization of conduction deficits by aldose reductase inhibitors does not have a vascular mechanism. Suggestions for an alternative mechanism may be found elsewhere.[42]

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